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A long non-coding RNA regulates cadherin transcription and susceptibility to Bt toxin Cry1Ac in pink bollworm, *Pectinophora gossypiella*

Shengyun Li[a,b,c,1], Fiaz Hussain[c,d,1], Gopalan C. Unnithan[c], Shuanglin Dong[a,*], Zain UlAbdin[d], Shaohua Gu[e], Lolita G. Mathew[f], Jeffrey A. Fabrick[f], Xinzhi Ni[g], Yves Carrière[c], Bruce E. Tabashnik[c], Xianchun Li[c,*]

[a] *Key Laboratory of Integrated Management of Crop Diseases and Pests (Ministry of Education), College of Plant Protection, Nanjing Agricultural University, Nanjing 210095, China*
[b] *School of Agricultural Sciences, Zhengzhou University, Zhengzhou 450001, Henan, China*
[c] *Department of Entomology, University of Arizona, Tucson, AZ 85721, USA*
[d] *Insect Molecular Biology Lab, Department of Entomology, University of Agriculture, Faisalabad 38040, Pakistan*
[e] *State Key Laboratory for Biology of Plant Diseases and Insect Pests, Institute of Plant Protection, Chinese Academy of Agricultural Sciences, Beijing 100193, China*
[f] *USDA, ARS, U.S. Arid Land Agricultural Research Center, Maricopa, AZ 85138, USA*
[g] *USDA, ARS Crop Genetics and Breeding Research Unit, Tifton, GA 31793, USA*

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ABSTRACT

Extensive planting of transgenic crops producing insecticidal proteins from the bacterium *Bacillus thuringiensis* (Bt) has spurred increasingly rapid evolution of resistance in pests. In the pink bollworm, *Pectinophora gossypiella*, a devastating global pest, resistance to Bt toxin Cry1Ac produced by transgenic cotton is linked with mutations in a gene (*PgCad1)* encoding a cadherin protein that binds Cry1Ac in the larval midgut. We previously reported a long non-coding RNA (lncRNA) in intron 20 of cadherin alleles associated with both resistance and susceptibility to Cry1Ac. Here we tested the hypothesis that reducing expression of this lncRNA decreases transcription of *PgCad1* and susceptibility to Cry1Ac. Quantitative RT-PCR showed that feeding susceptible neonates small interfering RNAs (siRNAs) targeting this lncRNA but not *PgCad1* decreased the abundance of transcripts of both the lncRNA and *PgCad1*. Moreover, neonates fed the siRNAs had lower susceptibility to Cry1Ac. The results imply that the lncRNA increases transcription of *PgCad1* and susceptibility of pink bollworm to Cry1Ac. The results suggest that disruption of lncRNA expression could be a novel mechanism of pest resistance to Bt toxins.

1. Introduction

Transgenic crops producing *Bacillus thuringiensis* (Bt) toxins are planted widely for control of lepidopteran and coleopteran pests (James, 2017). Binding of Bt toxins to their target site receptors, which are anchored to the apical membrane of the midgut cells, is a key step leading to death of pest larvae (Adang et al., 2014; Pardo-López et al., 2013). Reduction of this binding is the most common mechanism of resistance to crystalline (Cry) Bt toxins (Jin et al., 2015; Tabashnik, 2015; Wu, 2014; Zhang et al., 2012). This mechanism occurs for all known receptors of Bt toxin Cry1Ac, including cadherin (Gahan et al., 2001; Wang et al., 2016), alkaline phosphatase (ALP) (Guo et al., 2015; Jurat-Fuentes and Adang, 2004), aminopeptidase N (APN) (Wang et al., 2017b) and ABC transporters (Gahan et al., 2010; Park et al., 2014; Wang et al., 2017a).

The pink bollworm (*Pectinophora gossypiella*) is a major pest of cotton worldwide (CABI, 2016. http:www.cabi.org/isc/datasheet/39417). Resistance of this pest to Cry1Ac and Cry1Ac-producing Bt cotton is conferred by various insertion and deletion mutations of the *P. gossypiella* cadherin gene (*PgCad1)*. Sixteen Cry1Ac resistance alleles of *PgCad1* have been identified: four (*r1-r4)* from lab-selected Arizona strains (Fabrick and Tabashnik, 2012; Morin et al., 2003), eight (*r5-r12)* from field-selected populations in India (Fabrick et al., 2014), and four from lab-selected strains in China (*r13-r16)* (Wang et al., 2018, 2019). Analysis of the *r3* allele, which lacks the entire 126 bp exon 21 in its cDNA, revealed that loss of exon 21 results from insertion in *PgCad1* of a 4739 bp intact chicken repeat 1 (CR1) retrotransposon named *CR1-1_Pg* (Fabrick et al., 2011). Unexpectedly, intron 20 of both *PgCad1*

* Corresponding authors.
E-mail addresses: lxc@email.arizona.edu (X. Li), sldong@njau.edu.cn (S. Dong).
[1] These authors contributed equally.

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susceptible (*s*) and resistant (*r1-r3*) alleles encodes an intronic sense lncRNA of ~ 400 bp named *PgCad1 lncRNA* (Fabrick et al., 2011). However, the effects of this lncRNA were previously unknown.

Three independent transcriptomics studies detected many lncRNA transcripts that differ in abundance between strains of the diamondback moth (*Plutella xylostella*) that are resistant or susceptible to Bt toxins or other insecticides (Etebari et al., 2015; Liu et al., 2017; Zhu et al., 2017). This led to speculation that these differences could affect susceptibility to Bt or other insecticides (Etebari et al., 2015; Liu et al., 2017; Zhu et al., 2017). Moreover, intronic lncRNAs often epigenetically regulate expression of their genomically associated protein-coding genes (Dinger et al., 2008; Guil et al., 2012; He et al., 2012; Long et al., 2017; Nakaya et al., 2007). Thus, we hypothesized that *PgCad1 lncRNA* regulates transcription of *PgCad1,* and thereby affects susceptibility of pink bollworm to Cry1Ac. To test this hypothesis, we fed susceptible pink bollworm neonates on diet treated with small interfering RNAs (siRNAs) targeting *PgCad1 lncRNA* but not *PgCad1*. Compared with controls, this treatment significantly reduced the abundance of transcripts of *PgCad1 lncRNA* and *PgCad1*, and also significantly decreased larval susceptibility to Cry1Ac. These results imply that *PgCad1 lncRNA* enhances *PgCad1* transcription and thereby increases pink bollworm susceptibility to Cry1Ac.

2. Materials and methods

2.1. Insects, siRNAs, Cry1Ac and bioassays

The susceptible APHIS-S strain of pink bollworm used in this study had been reared on wheat germ diet (Bartlett and Wolf, 1985) in the laboratory for > 35 years without exposure to toxins (Liu et al., 2001). MVP II (Dow Agrosciences, San Diego, CA), a liquid formulation containing a hybrid protoxin identical to the holotype Cry1Ac protoxin in its first 1067 of 1182 amino acids including the entire active toxin (domains I, II, and III) (Fabrick et al., 2015), was used as the source of Cry1Ac. Two siRNAs (siRNA1 and siRNA2) of *PgCad1 lncRNA* (Table 1 and Fig. 1) targeting *PgCad1 lncRNA* but not *PgCad1,* and one negative control siRNA (ncsiRNA) targeting neither *PgCad1 lncRNA* nor *PgCad1* were designed and synthesized by RiboBio (Guangzhou, China). All three siRNAs were 2′-*O*-methyl-modified to increase their potency and stability and to minimize their potential off-target effects (Jackson and Linsley, 2010).

We dissolved the three siRNAs in DEPC water to prepare a 16.7 μM solution of each siRNA. Three siRNA diets were prepared (104.4 pmol siRNA per mL diet, determined based on a preliminary experiment) by mixing 64 mL wheat germ diet with 400 μL of each siRNA solution (16.7 μM) and 6.4 μL dye in a benchtop mixer. Green food dye was added to confirm that the added siRNA (or MVP II for preparation of Cry1Ac diet below) was evenly mixed with the diet. Aliquots of each siRNA diet were dispensed into 80 wells (0.8 mL diet per well) of a 128-well bioassay tray (Bio-Serv, Frenchtown, New Jersey, USA). Diet treated with 0.9 μg Cry1Ac per mL diet or without Cry1Ac was prepared by mixing MVP II (dissolved in Na_2CO_3 buffer) or equal volume of Na_2CO_3 buffer into the wheat germ diet. The Cry1Ac and control diets were transferred into wells of 128-well bioassay trays in 1.0 mL aliquots. We used 0.9 μg Cry1Ac per mL diet, which killed 70% of APHIS-S neonates in a 21-day pilot bioassay. We expected growth inhibition but little or no mortality in the 8-day bioassays conducted here after prefeeding with siRNA, because of the shorter duration and testing of 4-day-old APHIS-S larvae rather than neonates on diet treated with Cry1Ac.

We transferred 480 neonates (< 5 h old) of the APHIS-S strain onto the three siRNA diet treatments (80 wells × 2 neonates/well = 160 neonates per siRNA diet). After feeding on siRNA diet for 4 days, 42 larvae (6 biological replicates of 7 larvae each) were randomly removed from each of the three siRNA diet treatments, flash-frozen in liquid nitrogen, and stored at −80 °C for subsequent RNA extraction and RT-qPCR analysis. Half (48 larvae) of the remaining larvae from each siRNA treatment were transferred individually onto control or Cry1Ac diet (1 larva per well). Thus, we tested six treatments (three siRNAs × two diets). Feeding on siRNA-treated diet and the subsequent diet bioassay were conducted at 27 ± 1 °C, 70% RH, and 16 L:8D. After 8 days, we recorded the number of survivors and their developmental stage and the weight of all survivors. Individuals that were either 4th instars or more mature (one individual pupated) were recorded as reaching 4th instar.

2.2. RNA extraction

We extracted six total RNA samples per siRNA treatment (each with 7,4 d old larvae) using the guanidinium-HCl procedure (Sambrook et al., 1989). We also prepared one total RNA sample with the same protocol from a pool of seven 4th instar larvae reared on control diet containing neither siRNA nor MVP II. We used the DNA-free™ DNA Removal Kit (Thermo Scientific, USA) to eliminate potential genomic DNA (gDNA) contamination in the 18 RNA samples (3 siRNAs × 6 replicates) and the control RNA sample. A PCR analysis of *EF-1α* in each purified RNA sample (with or without reverse transcription into cDNA) was conducted with the primers EF-1α-F and EF-1α-R (Table 1) to

Table 1
Primers and siRNAs used for cloning and expression analysis of pink bollworm *PgCad1 lncRNA* and *PgCad1*.

Primer	Sequence (5′-3′)	Amplification efficiency
GSP1	ACACCTTGCGGAATGACGTAGT	N.A.
Racer 3′ nested	CGCTACGTAACGGCATGACAGTG	
GSP2	CTATAACACCTTGCGGAATGACG	N.A.
Racer 3′	GCTGTCAACGATACGCTACGTAACG	
lncRNA-F	ATTGCTATCCATAATTCTAGGC	N.A.
lncRNA-R	TTCAGAATCAGAATCATTTATT	
Q-lncRNA-F	TTTCTTGTCATTTCTTCTCCTCAGC	92.4%
Q-lncRNA-R	CAGAATCAGAATCATTTATTCAACGTAAT	
Q-cadherin-F	GAACCAGACATTCGCCAT	96.3%
Q-cadherin-R	CGGTCCGTTGCTATTACCTT	
RPS7-F	CCGTGAGTTGGAGAAGAA	92.9%
RPS7-R	AGGATAGCGTCGTACACTGA	
EF-1α-F	GAAGTCAAGTCCGTGGAGATG	89.5%
EF-1α-R	GACCTGTGCTGTGAAGTCG	
siRNA1 sense	GCCTGGTATTCAATGTGTT	N.A.
siRNA2 sense	GCGGAATGACGTAGTTTCA	N.A.
ncsiRNA sense	siM12921102701 (Kept confidential according to RiboBio terms)	N.A.

N.A.: Not applicable.

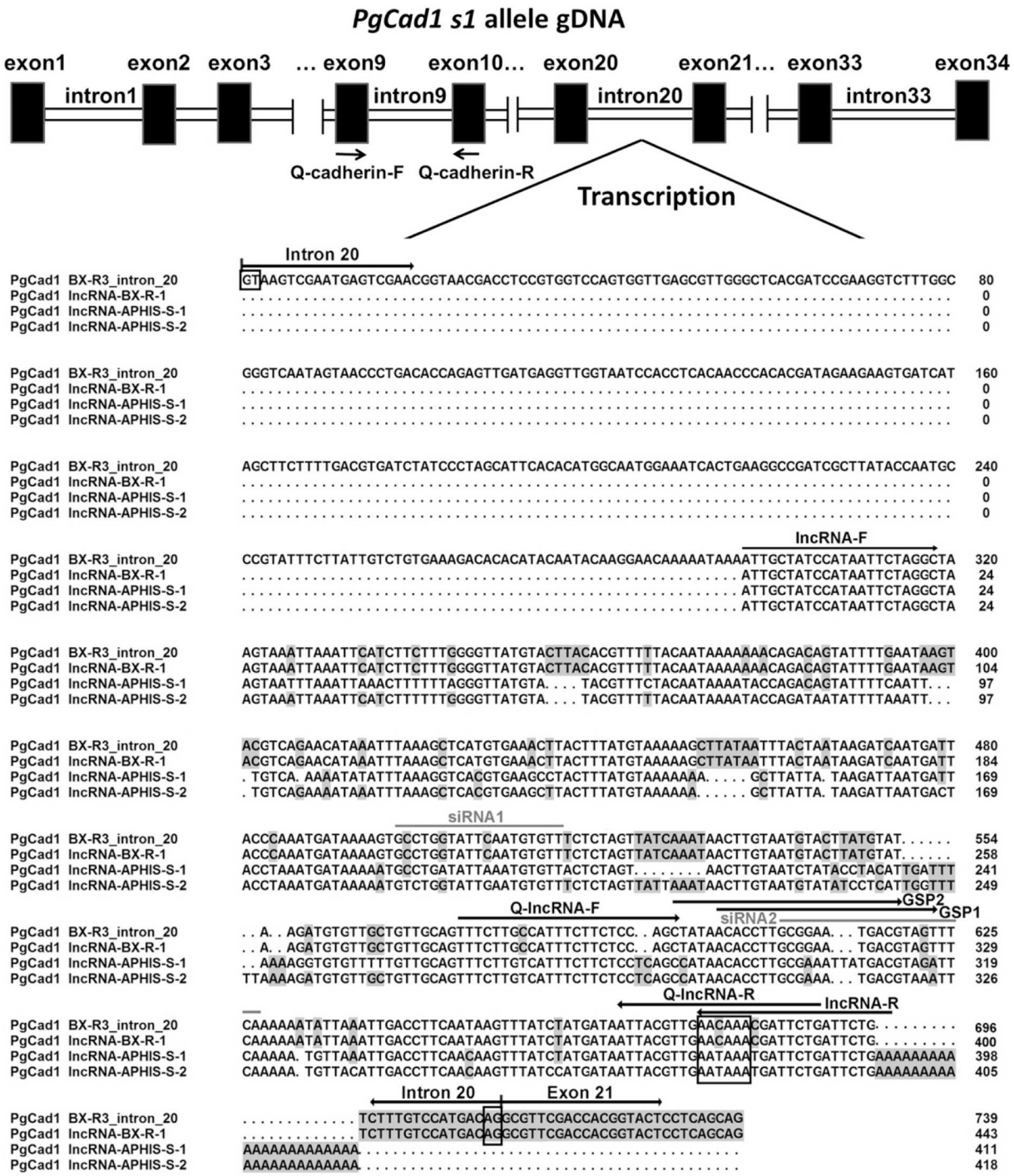

Fig. 1. Genomic structure of *PgCad1 s1* allele and alignment of *PgCad1 lncRNA* with *PgCad1* intron 20. The top schematic diagram depicts the genomic structure of *PgCad1 s1* allele. The alignment below the diagram contains the intron 20 of the *PgCad1 r3* allele from BX-R strain and 3 *PgCad1 lncRNA* sequences transcribed in the sense orientation from the intron 20 of different *PgCad1* alleles. All the indels (insertions/deletions) and SNPs (single nucleotide polymorphisms) among the 4 sequences are shaded in gray. The 5′ GT and 3′ AG dinucleotide splice sites of the *PgCad1* intron 20 and the polyadenylation signal hexamer AAT(C)AAA are boxed. The annealing directions and positions of the primers used for the 3′ RACE and full-length cDNA cloning of *PgCad1 lncRNA* from APHIS-S strain as well as for RT-qPCR analyses of *PgCad1* and *PgCad1 lncRNA* are depicted with arrows (in the diagram) or arrowed lines (in the alignment) and the corresponding primer names. The target regions of the two siRNAs of *PgCad1 lncRNA* (siRNA 1 and siRNA2 in Table 1) are indicated with two gray lines.

confirm the absence of gDNA. RNA concentrations were determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific, USA).

2.3. RT-PCR cloning and sequencing of PgCad1 lncRNA full-length cDNA

Because the 5′-end of the *PgCad1 lncRNA* cDNA was previously determined from its chimeric transcript with *CR1-1_Pg* (Fabrick et al., 2011), cloning of the full-length *PgCad1 lncRNA* cDNA sequence from the APHIS-S strain began with a 3′-Rapid Amplification of cDNA Ends (3′-RACE) using two sense gene-specific primers (GSP1 and GSP2) with two GeneRacer™ antisense primers (Thermo Scientific, USA) (Table 1). For 3′-RACE, the cDNA reverse transcribed from 1 μg of purified RNA of the control larvae with the Gene Racer™ Oligo-dT primer using Superscript III RT Module of the Gene Racer kit (Thermo Scientific, USA)

was used as the template for the initial touchdown PCR with the Gene Racer™ 3′ primer and GSP2. The touchdown PCR reaction was initiated with 2 min denaturation at 94 °C, followed by 13 cycles of 94 °C for 15 s, 74 °C for 15 s (−1 °C per cycle increment) and 72 °C for 30 s, 16 cycles of 94 °C for 15 s, 62 °C for 15 s and 72 °C for 30 s, and a final elongation for 2 min at 72 °C in an Eppendorf Master Cycler. One μL of the touchdown PCR product was used as the template for the subsequent nested PCR with GSP1 and the Gene Racer™ 3′ nested primer. The nested PCR cycling conditions included 2 min denaturation at 94 °C, followed by 30 cycles of 94 °C for 15 s, 62 °C for 15 s and 72 °C for 30 s, and a final elongation for 2 min at 72 °C. The final full-length PCR used the same control larvae cDNA as the template and conducted with 3 min denaturation at 94 °C, followed by 35 cycles of 94 °C for 30 s, 51 °C for 30 s and 72 °C for 1 min, and a final 10 min extension at 72 °C.

Both the 3′-RACE and full-length PCR products were separated on 1% agarose gels, visualized by ethidium bromide staining, gel-purified with QIAquick Gel Extraction Kit (Qiagen, CA), cloned into pGEM®-T Easy Vector (Promega, USA) and sequenced by the Genomic Analysis & Technology Core Facility of the University of Arizona. The sequences of two *PgCad1 lncRNA* alleles obtained from the susceptible APHIS-S strain were aligned with the 5′ lncRNA portion (GenBank Acc. No. HQ845204.1) of the chimeric transcript (Fabrick et al., 2011) and the intron 20 and exon 21 of the *PgCad1 r3* allele (HQ585015.1) from the resistant BX-R strain using DNAMAN version v6 software.

2.4. RT-qPCR analyses of PgCad1 lncRNA and PgCad1 expression

We reverse transcribed 1.2 μg of each siRNA-treated larval RNA sample into cDNA with oligo(dT)$_{20}$ primer and SuperScript III Reverse Transcriptase (Invitrogen, USA). Quantitative RT-PCR (RT-qPCR) of *PgCad1 lncRNA, PgCad1*, and the two internal reference genes *RPS7* and *EF-1α* were performed individually in a 25 μL reaction containing 12.5 μL 2× Maxima SYBR Green/ROX RT-qPCR Master Mix (Thermo Scientific, USA), 7.5 pmol gene-specific primer pair (0.75 μL of 10 μM primer solution each; Table 1), 0.8 μL cDNA and 10.2 μL nuclease-free water using a CFX Connect Real-Time PCR Detection System (Bio-Rad, USA). The RT-qPCR of each of the four genes began with an initial denaturation at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s, annealing at 56 °C for 30 s and extension at 72 °C for 30 s, during which real-time data were collected. Melting curve analysis from 65 °C to 95 °C was performed for each target and reference gene to ensure amplification of single amplicons. We used a five-fold serial dilution of each cDNA sample as the template to produce a log template concentration (x-axis) - Cq value (y-axis) line for each of the four genes. The slopes of the four lines obtained were used to estimate the amplification efficiency (E; see Table 1) of each gene using the formula $E = 10^{-1/\text{slope}} - 1$ (Bustin et al., 2009). The expression level of each gene was then calculated with their mean Cq and amplification efficiency (Eq. 1). The expression levels of the two target genes (*PgCad1 lncRNA* and *PgCad1*) were normalized with the geometric mean of the expression of the two reference genes (*RPS7* and *EF-1α*) (Eq. 2) (Livak and Schmittgen, 2001; Vandesompele et al., 2002).

$$\text{Expression level} = (1 + E)^{-Cq} \quad (1)$$

$$\text{Normalized expression level of each target gene} = \frac{(1 + E_{target\ gene})^{-Cq_{target\ gene}}}{\sqrt{(1 + E_{EF-1\alpha})^{-Cq_{EF-1\alpha}} \times (1 + E_{RPS7})^{-Cq_{RPS7}}}} \quad (2)$$

2.5. Statistical analysis

We used *a priori* contrasts to separately compare effects of siRNA1 vs. ncsiRNA and siRNA2 vs. ncsiRNA. For abundance of *PgCad1 lncRNA* and *PgCad1* transcript abundance, contrasts followed one-way ANOVA. For percentage of larvae reaching 4th instar, contrasts followed logistic regression for binary data. For larval weight, contrasts followed two-way ANOVA of log-transformed data. We used one-sided *P* values to test the 1-sided hypothesis that either siRNA1 or siRNA2 significantly reduced susceptibility to Cry1Ac.

3. Results

3.1. PgCad1 lncRNA sequences

We used 3′-RACE and the nested gene-specific primers GSP1 and GSP2 (Table 1 and Fig. 1) to determine the 3′ end of the pure *PgCad1 lncRNA* transcript from the susceptible APHIS-S strain. These primers are based on the partial *PgCad1 lncRNA* transcript from the *r3* cadherin allele resistant strain BX-R (Genbank Acc. No. HQ845204; Fabrick et al., 2011). We amplified the full-length cDNA of this lncRNA using RT-PCR with the primers lncRNA-F and lncRNA-R (Table 1 and Fig. 1), which were based on both the BX-R strain *PgCad1 lncRNA* transcript (Fabrick et al., 2011) and the 3′ RACE product sequence. The resultant full-length RT-PCR products were TA-cloned into pGEM®-T easy vector. Sequencing of ten positive clones yielded two pure *PgCad1 lncRNA* full-length cDNA sequences. We named the two APHIS-S *PgCad1 lncRNA* sequences *PgCad1 lncRNA-APHIS-S-1* (from seven clones) and *PgCad1 lncRNA-APHIS-S-2* (from three clones; GenBank Acc. Nos. MG839502 and MG839503, respectively). We named the BX-R strain *PgCad1 lncRNA* transcript *PgCad1 lncRNA-BX-R-1*.

We aligned the cDNA sequences of the three *PgCad1 lncRNA* alleles with the *PgCad1 r3* allele intron 20 and exon 21 gDNA sequence from the BX-R strain (GenBank Acc. No. HQ585015.1) (Fig. 1). The alignment shows that transcription of *PgCad1 lncRNA-APHIS-S-1* and *PgCad1 lncRNA-APHIS-S-2* is initiated 297 bp downstream of the 5′ GT dinucleotide splice site of the *PgCad1* intron 20, and polyadenylated 14 bp downstream of the canonical polyadenylation signal AAUAAA and 14 bp upstream of the 3′ AG dinucleotide splice site of the *PgCad1* intron 20. Although transcription of *PgCad1 lncRNA-BX-R-1* is initiated at the same position as that of the two APHIS-S lncRNAs, it fails to terminate/polyadenylate within the *PgCad1* intron 20, likely due to mutation of the polyadenylation signal AAUAAA to AACAAA (Fig. 1) and/or the insertion of a chicken repeat 1 retrotransposon (*CR1-1_Pg*) in the *PgCad1* exon 21 (Fabrick et al., 2011). *PgCad1 lncRNA-APHIS-S-1* and *PgCad1 lncRNA-APHIS-S-2* are 411 and 418 bp long, respectively, and 93% identical to each other. The differences include five small indels (insertions/deletions) of 1, 1, 2, 3 and 8 bp (Fig. 1), respectively. The two APHIS-S lncRNAs share 80% and 83% identity with *PgCad1 lncRNA-BX-R-1*, respectively.

3.2. Suppressing PgCad1 lncRNA significantly reduced PgCad1 transcription

To observe effects of *PgCad1 lncRNA* on *PgCad1* transcription, we used RNA interference (RNAi) to knockdown *PgCad1 lncRNA* in APHIS-S. Neonates were fed for 4 d on diet containing small interfering RNA 1 or 2 (siRNA1 or siRNA2) of *PgCad1 lncRNA* (Table 1 and Fig. 1) or negative control siRNA (ncsiRNA), each at 104.4 pmol siRNA per mL diet. Relative to ncsiRNA, both siRNA1 and siRNA2 significantly reduced *PgCad1 lncRNA* transcript abundance (siRNA1: 37% reduction, $F_{1,15} = 10.20$, $P = .0061$; siRNA2: 45% reduction, $F_{1,15} = 15.25$, $P = .0014$) (Fig. 2A). In addition, relative to ncsiRNA, both siRNA1 and siRNA2 significantly reduced *PgCad1* transcript abundance (siRNA1: 37% reduction, $F_{1,5} = 6.51$, $P = .022$; siRNA2: 68% reduction, $F_{1,15} = 22.49$, $P = .0003$) (Fig. 2B).

3.3. Suppressing PgCad1 lncRNA decreased pink bollworm susceptibility to Cry1Ac

In the 8-day bioassays initiated with 4-day-old larvae that had been pre-fed siRNAs, diet treated with 0.9 μg Cry1Ac per mL diet caused no

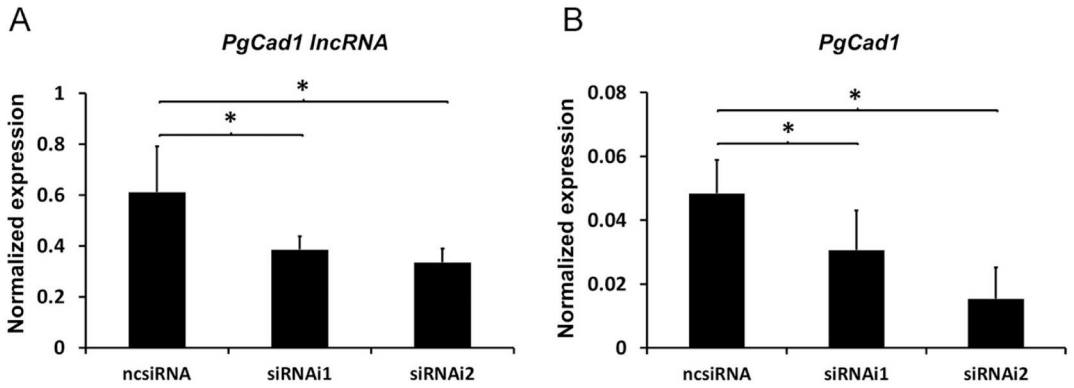

Fig. 2. Effects of two *PgCad1 lncRNA* siRNAs on the transcription levels of *PgCad1 lncRNA* (A) and *PgCad1* (B). The bars show the mean and standard error based on six biological replicates of 7 susceptible larvae for each siRNA treatment after 4 d. Significant differences between the larvae fed ncsiRNA (negative control siRNA) and those fed siRNA1 or siRNA2 of *PgCad1 lncRNA* are indicated by asterisks ($P < .05$, One-way ANOVA followed by *a priori* contrasts).

mortality but inhibited larval growth. On the treated diet, the percentage of larvae reaching fourth instar was 37% for larvae that had been pre-fed ncsiRNA. Relative to larvae pre-fed ncsiRNA, the percentage of larvae reaching fourth instar on treated diet was significantly higher for larvae that had been pre-fed siRNA2 (58%) (1.6× increase, $\chi^2 = 3.44$, one-sided $P = .032$), but not siRNA1 (51%) ($\chi^2 = 1.50$, one-sided $P = .11$) (Fig. 3A). On the treated diet, larval weight was significantly higher for larvae pre-fed siRNA1 (4.32 mg) ($F_{1,\ 222} = 2.86$, one-sided $P = .046$) or siRNA2 (4.91 mg) ($F_{1,222} = 9.15$, one-sided $P = .0014$) relative to larvae pre-fed ncsiRNA (3.92 mg) (Fig. 3B). By contrast, on control diet, significant differences did not occur between ncsiRNA and either siRNA1 or siRNA2 in the percentage of larvae reaching fourth instar (96% for ncsiRNA, 96% for siRNA1, and 98% for siRNA2; $P > .19$ for each comparison) (Fig. 3A) or larval weight (22.0 mg for ncsiRNA, 23.8 mg for siRNA1, and 22.2 mg for siRNA2; $P > .50$ for each comparison) (Fig. 3B).

4. Discussion

The pink bollworm gene *PgCad1* encodes a cadherin protein that binds Cry1Ac in the larval midgut, which is essential for toxicity (Fabrick and Wu, 2015). We previously found that *PgCad1* also encodes a lncRNA (i.e., *PgCad1 lncRNA*) that yields a chimeric transcript with the downstream chicken repeat retrotransposon (*CR1-1_Pg*) inserted into exon 21 of *PgCad1 r3* allele in the Cry1Ac-resistant BX-R strain (Fabrick et al., 2011). Here we obtained two full-length native transcripts of *PgCad1 lncRNA* from the Cry1Ac-susceptible APHIS-S strain corresponding to wild-type (i.e., susceptible) *PgCad1* alleles. Both of these *PgCad1 lncRNA* transcripts start 297 bp downstream of the 5′ splice site of intron 20 of *PgCad1* and polyadenylate 14 bp upstream of the 3′ splice site of intron 20 (Fig. 1). This indicates that *PgCad1 lncRNA* is an intronic sense lncRNA without introns. Although a previous report showed that a lncRNA overlaps with intron 8 in the rainbow trout protocadherin 8 (*pcdh8*) in sense orientation (Paneru et al., 2016), we are not aware of any previous reports of lncRNAs encoded by cadherin loci in other animals including mammals.

On untreated control diet, pre-feeding susceptible larvae with either one of two siRNAs of *PgCad1 lncRNA* (siRNA1 and siRNA2) reduced transcript abundance of this lncRNA (Fig. 2A), but had no significant effect on larval development or weight relative to the negative control ncsiRNA on control diet (Fig. 3A and B). These two siRNAs also significantly reduced abundance of the transcripts of *PgCad1* (Fig. 2B). This can happen only if 1) *PgCad1 lncRNA* endogenously acts to positively regulate transcription of *PgCad1*; and/or 2) the seed regions (position 2–8) of the guide strands of siRNA1 and siRNA2 are complementary to the 3′ UTR of *PgCad1* cDNA sequence (Jackson et al., 2006; Lin et al., 2005; Jackson and Linsley, 2010), turning *PgCad1* into an off-target gene of the two siRNAs. Alignments of siRNA1 and siRNA2 with the *PgCad1* cDNA sequence detected six short matches of 7 or 8 bp for siRNA1 and four short matches of 7 or 8 bp for siRNA2, but none meet the requirement of sequence complementarity between the seed region of siRNA1 or siRNA2 and the 3′ UTR of *PgCad1* (Table 2). Furthermore, both siRNA1 and siRNA2 were 2′-*O*-methyl-modified to minimize their potential off-target effects (Jackson and Linsley, 2010). Moreover, the direct silencing effects of siRNAs are usually greater for target genes than off-target genes (Lin et al., 2005; Jackson et al., 2006;

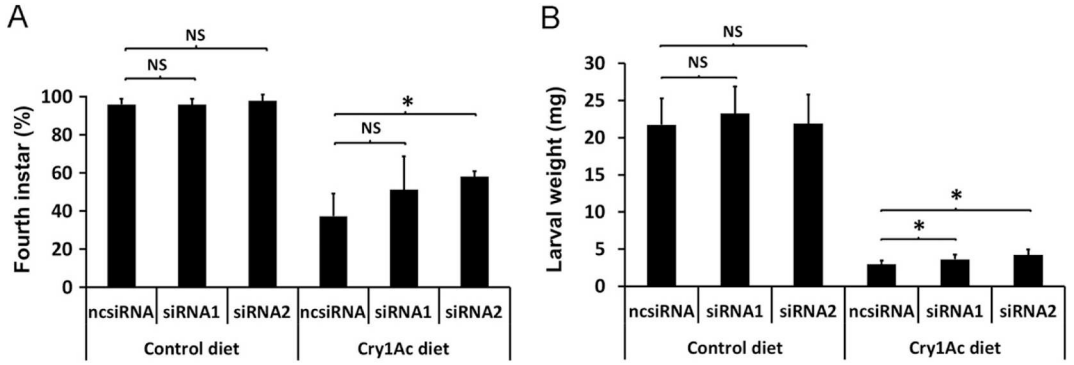

Fig. 3. Effects of *PgCad1 lncRNA* siRNAs on pink bollworm susceptibility to Cry1Ac. A. Larvae reaching fourth instar (%). Means and standard errors shown are based on three replicates of 16 larvae for each siRNA treatment. B. Weight of survivors. Back-transformed means are shown with 95% confidence intervals. Significant differences in the percentage of fourth instars (logistic regression for binary data followed by contrasts) and the weight of survivors (log-transformed before analysis, two-way ANOVA followed by contrasts) between the APHIS-S larvae fed ncsiRNA (negative control siRNA) and those fed siRNA1 or siRNA2 of *PgCad1 lncRNA* are indicated by asterisks (one-sided $P < .05$). NS = no significant difference.

Table 2
Sequence conservation between the guide strands of siRNA1 and siRNA2 of *PgCad1 lncRNA* with the *PgCad1* cDNA sequence.

siRNA guide strand alignment to *PgCad1* cDNA[b]	Sequence conservation[a]	
	Seed region[c]	3′ UTR[d]
siRNA1 2 ACACATTGA 10	Yes	No
PgCad1 102 ACACATTGA 110		
siRNA1 4 ACATTGA 10	No	No
PgCad1 748 ACATTGA 754		
siRNA1 4 ACATTGA 10	No	No
PgCad1 2599 ACATTGA 2605		
siRNA1 9 GAATACCA 16	No	No
PgCad1 4246 GAATACCA 4253		
siRNA1 10 AATACCA 16	No	No
PgCad1 1719 AATACCA 1725		
siRNA1 10 AATACCA 16	No	Yes
PgCad1 5471 AATACCA 5477		
siRNA2 3 AAACTAC 9	No	No
PgCad1 3788 AAACTAC 3794		
siRNA2 8 ACGTCAT 14	No	No
PgCad1 4558 ACGTCAT 4564		
siRNA2 11 TCATTCCG 18	No	No
PgCad1 4391 TCATTCCG 4398		
siRNA2 12 CATTCCGC 19	No	No
PgCad1 880 CATTCCGC 887		

[a] Only complementary short matches of 7 or 8 bp located in the seed region (position 2–8) of either siRNA and the 3′ UTR of *PgCad1* qualify *PgCad1* to be an off-target of that siRNA (Jackson et al., 2006; Jackson and Linsley, 2010).

[b] siRNA guide strand is complementary to the *PgCad1* cDNA. Numbers indicate the start and end positions within the siRNA guide strand and *PgCad1* cDNA.

[c] Seed region is the sequence from position 2–8 at the 5′ end of guide strand implicated in target recognition. “Yes” or “No” responses indicate if sequence conservation is within the seed region of the siRNA.

[d] 3′ UTR corresponds to the 3′ untranslated region of *PgCad1*. “Yes” or “No” responses indicate if sequence conservation is within the 3′ UTR of the *PgCad1* cDNA.

Li et al., 2015). Yet, here the silencing efficiency was not greater for the target gene *PgCad1 lncRNA* (37% and 45% by siRNA1 and siRNA2, respectively) than for the off-target gene *PgCad1* (37% and 68% by siRNA1 and siRNA2, respectively) (Fig. 2). This confirms that *PgCad1 lncRNA* increases transcription of *PgCad1* and that *PgCad1* transcription was not affected directly by siRNA1 or siRNA2. Although this is the first report of lncRNA regulation of cadherin in insects, human E-cadherin is known to be positively or negatively regulated by multiple human lncRNAs (Han et al., 2016; Luo et al., 2013; Sun et al., 2014a; Sun et al., 2014b; Wu et al., 2015; Yang et al., 2011; Yuan et al., 2014; Zhang et al., 2018).

Although both siRNAs significantly knocked down *PgCad1 lncRNA,* siRNA2 silenced 8% more of *PgCad1 lncRNA* transcript than did siRNA1 (Fig. 2A) and siRNA2 caused 31.5% more reduction in *PgCad1* expression than did siRNA1 (Fig. 2B). Also, on diet treated with Cry1Ac, both siRNA1 and siRNA2 significantly increased larval weight relative to the negative control ncsiRNA, but the increase in the percentage of larvae reaching fourth instar was statistically significant for siRNA2 (a 1.6-fold increase) but not for siRNA1 (a 1.4-fold increase) (Fig. 3). In the alignment with *PgCad1 lncRNA-APHIS-S-1*, the predominant allele (7 of the 10 clones) in the APHIS-S strain, siRNA2 has a 3 bp deletion, but siRNA1 does not (Fig. 1). Thus, siRNA1 is more similar than siRNA2 to *PgCad1 lncRNA-APHIS-S-1*. This suggests that the different target regions of the two siRNAs, rather than their sequence similarity with the predominant allele, account for the greater effects of siRNA2 relative to siRNA1.

Although no mortality occurred in any treatment in the 8-day bioassays initiated with larvae that had been pre-fed siRNAs for 4 days, feeding on diet treated with 0.9 µg Cry1Ac per mL diet inhibited larval growth. Moreover, the reduction in growth inhibition caused by pre-feeding with siRNA1 and siRNA2 noted above is expected to substantially increase fitness. In particular, we would expect relatively high mortality before reaching the adult stage for larvae that did not reach fourth instar after 12 days (including 4 days pre-feeding on siRNAs and the subsequent 8-day bioassay). Thus, the 1.5-fold higher mean percentage of larvae reaching fourth instar for larvae pre-fed with siRNA1 and siRNA2 (relative to ncsiRNA) is expected to substantially boost fitness.

The finding that knockdown of *PgCad1 lncRNA* repressed *PgCad1* transcription and significantly decreased susceptibility to Cry1Ac (Fig. 3) suggests that *PgCad1 lncRNA* acts as a positive regulator to enhance the expression of its genomically associated cadherin, and thus increase susceptibility to Cry1Ac in pink bollworm larvae. Any mutations that disrupt the expression or function of *PgCad1 lncRNA* in pink bollworm or similar lncRNAs in other insects could reduce cadherin expression and thereby cause reduced susceptibility to Cry1Ac. Although previous genomics results suggested an association between lncRNA abundance and resistance to Bt or other insecticides in diamondback moth (Etebari et al., 2015; Liu et al., 2017; Zhu et al., 2017), our results provide direct evidence for the involvement of lncRNA in reducing susceptibility to a Bt toxin. Considered together with the previously reported genomics data, our results suggest that lncRNA-mediated resistance could be an important, novel mechanism of insect resistance to natural and synthetic insecticides.

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